SOME HISTOCHEMICAL STUDIES
OF
THE MOTOR ENDPLATE

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1965

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THE MOTOR ENDPLATE

Submitted in partial fulfillment for the degree of
Doctor of Medicine
Yale University
April 15, 1965
By Margretta Reed Seashore
B.A. Swarthmore College, 1961

With grateful appreciation to

Dr. Gilbert H. Glaser

without whose patient help and generosity
this work could not have been done.

I. INTRODUCTION

A. The Motor End Plate

The motor end plate has been under investigation for nearly 137 years, thus predating the cellular theory of tissue organization. The concepts of the junction of nerve and muscle have changed with the advance of knowledge in neurology and neuroanatomy and the development of newer techniques. The evolution of the modern concept of this important anatomic and physiologic junction can be traced from early studies. (49).

The first published investigations are those of Valentin and Emmert in 1836. They described an "arc-like" ending of the motor nerve at the muscle, and concluded that the motor nerve progressed from the central nervous system to the muscle, continued around the arc, and then returned to the central nervous system. They postulated a continuous stream of "nervous fluid" in the motor nerve.

Doyere in 1840 was the first to propose that the motor nerve came to a complete end at the muscle. This was confirmed in 1843 by Quartrefages and by Wagner in 1847, who also noted that the myelin sheath of the nerve was lost shortly before its entry into the muscle.

No more advances were made until 1862 when Kuhne, using gold staining, showed branching and complete arborization at the nerve terminal. In the same year, Rouget

demonstrated a heap of granules at the nerve terminal, and recognized the specialized nature of the neuromuscular junction.

Ranvier, in 1878, began using a gold impregnation technique, and just after the turn of the century, Cajal and others introduced the still-used silver staining technique. In 1863, Krause first used the term "motorische end plate" in his studies of the cat eye retractor muscle.

The first descriptions of the motor end plate are those obtained with gold impregnation. These show terminal nerve endings and some fringe-like structures. Silver staining (of which there are a number of techniques) reveals the more delicate structure of the neurofibrils, boutons termineaux, and granules at the nerve endings. The chemical nature of the reaction is not known, but a silver-protein complex is proposed. Methylene blue has been used to elucidate the structure of the motor end plates. Staining with it reveals terminal axons with arborizations, and some other structures, but again the nature of the reaction is not known. Numerous descriptions of fine detail of motor end plate structures are available, all somewhat dependent on the technique used. The advent of the electron microscope has allowed study of even finer structural detail (4). With this tool, the details of the anatomic interrelation of nerve and muscle are being seen.

Motor end plates are not distributed randomly among muscle fibers, but rather are distributed in well-localized bands, usually near the middle of the muscle (Figure 1). Commonly there is only one end plate per muscle fiber, although about 2.3% of fibers in normal human muscle have two end plates (9, 10). These are most commonly from the same nerve fiber or the result of terminal branching. Occasionally one end plate is supplied by two different nerve fibers. One end plate supplied by two different axons has not been observed (49).

There is a variation in shape and size of the motor end plate from muscle to muscle in the same animal and among different animals. The variations in end plate size seem to parallel the variation in muscle fiber size within a given muscle (10). Slow reacting ("red") muscles have larger end plates (10).

In addition to descriptions of normal muscle, many changes in the diseased muscle have been described using all of the techniques mentioned. The three principal pathologic changes are:

1. change in end plate size
2. degenerative changes in fibers and endings
3. reactive sprouting of nerve endings

The change in the size of the end plate follows the change in the size of the muscle fiber. Immature end plates may be larger or smaller than normal. In thyrotoxic myopathy

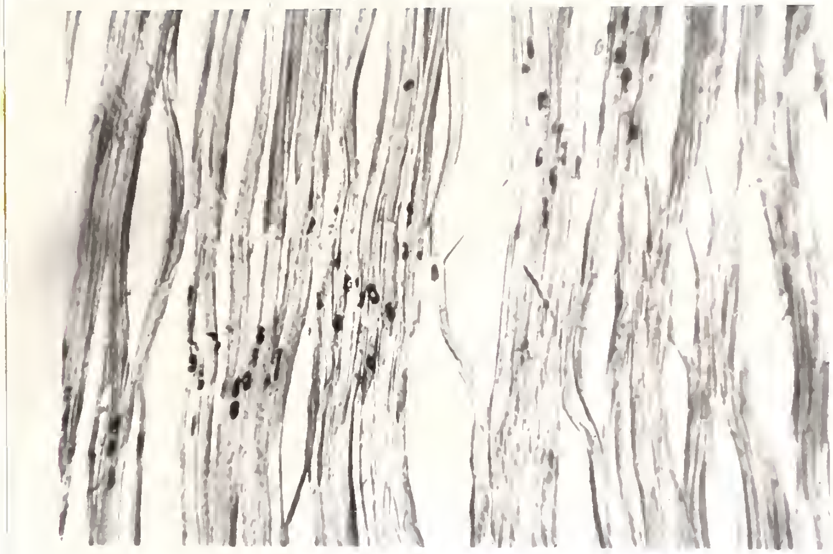


Fig.1. Distribution of endplates in normal mouse muscle. 50x

the formation of multiple end plates on one muscle fiber occurs. There are well-formed but enlarged motor end plates in upper motor neuron disease (10).

All of the early work, of course, focused on the anatomy of the motor end plate, and studies of the function awaited the development of more refined biochemical tools.

Loewi's discovery of "vagusstuff" began the study of neurohumoral transmitters. Then, in 1926, Loewi demonstrated an enzyme which destroyed the "vagusstuff" he had earlier discovered.

In 1940, Couteaux and Nachmannsohn demonstrated a correlation between the number of end plates in a given muscle and the amount of Loewi's enzyme, acetylocholinesterase, as demonstrated by microchemical techniques. Nachmannsohn also showed that the acetylcholinesterase present at the motor end plate in frog muscle was enough to destroy the calculated amount of acetylocholine during the refractory period of the muscle. He followed this with the microchemical demonstration that the highest concentration of acetylcholinesterase present in the muscle was in the motor end plate region (49).

Histochemical methods for localization of this enzyme in motor end plates have been developed. These will be discussed in detail later.

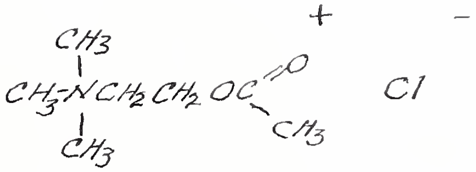
Thus the foundations for the synaptic theory of the function of the motor end plate were laid. The anatomic structure was demonstrated, although incompletely. A physiologic

role for acetylcholine (Figure 2) as the biochemical transmitter, and acetylcholinesterase as the destructive enzyme was suggested. The theory could then be proposed that activity in the motor nerve somehow resulted in the production of a compound (acetylcholine) at the nerve terminal which in turn stimulated the muscle on the other side of the junction to activity. An enzyme to destroy the acetylcholine was present, and thus its action could be terminated. Acetylcholine depolarizes a receptor substance on the muscle side of the myoneural junction. The depolarization is brief, for the action of acetylcholinesterase is rapid. The receptor substance is generally thought to be an anionic receptor for the cationic head of acetylcholine, and an esteratic site for the acetyl group of acetylcholine.

B. Hereditary Myopathy in the Mouse

In 1955, Michelson, et al, described a strain of mice discovered at the Jackson laboratory, which was apparently an inbreeding mutation. These mice showed the clinical appearance of a kind of muscular dystrophy (35). Their clinical course was characterized by progressive ataxia, atrophy, and paralysis, along with subnormal weight. The posterior limbs were most affected. A kyphosis resulting from the procession of the atrophy and paralysis was seen. The animals were cachectic and died at an early age, usually between one and six months.

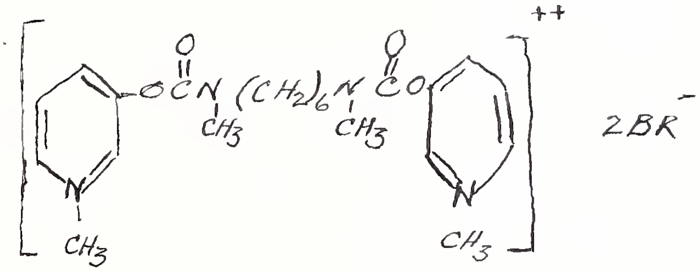
FIG.2.



$CH_3-\overset{CH_3}{\underset{CH_3}{N}}CH_2CH_2OC\overset{O}{\underset{CH_3}{}}\ \overset{+}{}\ Cl^{-}$

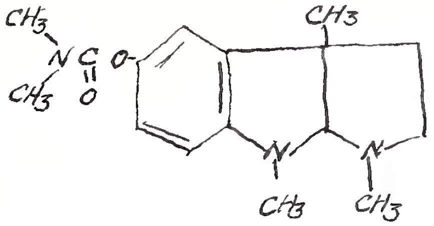
ACETYLCHOLINE (AS CHLORIDE)

FIG.3



BC 51

FIG. 4



PHYSOSTIGMINE

The microscopic studies done, failed to reveal any lesions in the central or peripheral nervous systems. Pathological changes were found only in muscle.

Later studies of the histopathology in these mice agreed with the findings described by Michelson and showed that although there was some variability among animals, certain lesions were common to all (45).

The major lesions seen were a variation in muscle fiber size, increase in endomysial connective tissue, coagulation necrosis of muscle fiber, subsarcolemmic nuclear accumulations, internal nuclear rowing and regenerative activity. There was an equal incidence of disease in the flexors and the extensors. Lesions in smooth and cardiac muscle were not seen. No sex differences were noted. On cross section of muscle, a variation in muscle fiber diameter was the most distinct change. There was a tendency toward rounding of the fibers.

There was much evidence for regeneration: nuclear rowing with large amounts of RNA, indicating anabolic activity, and the presence of myoblast-like cells. Hypertrophy of fibers was seen, but not swelling.

Other possible causes for nuclear rowing should be mentioned. Among them are failure to mature, atrophy or dedifferentiation, regenerative activity, reparative reaction to sublethal injury.

The mice are the result of an inbreeding mutation in the Jackson laboratory strain 129. Inheritance of the disease follows the pattern of an autosomal recessive (41, 45). The genetics have been studied by an unusual method of breeding which was developed because the female mice are not healthy enough to carry many pregnancies. Breeding is accomplished by ovarian transplant. The operation is done by grafting one half of the donor (dystrophic) ovary into the capsule of one of the recipient's ovaries after removal of both of the recipient's ovaries. Fertility following surgery is good and the healthy females are well able to carry several pregnancies.

Strain 129/Re- +dy is the subline of strain 129 in which the mutation arose. There are three genetic classes of animals from non-sibling matings of 129/Re- +dy animals:

1. dydy -- dystrophic animals
2. Dydy -- non-dystrophic carriers
3. Dydy -- either from carrier matings or from subline 129/J. All are non-dystrophic, non-carrier animals, and are used as controls in studies of the disease.

No close association of age or weight of animals with gross symptoms of dystrophy could be found (44).

A number of studies of the chemical constituents of dystrophic muscle as compared to normal muscle have been done.

There is increased lipid (34) along with decreased protein, decreased nitrogen, and increased collagen per gram of dry weight (44). Decreased creatinine has been reported by Kandeutsch, et al (27). Phospholipid concentration is the same in dystrophics as normal (48). Study of electrolyte concentration in muscle (2, 48) has revealed increased sodium and decreased potassium except for normal potassium in cardiac muscle.

Electrical profiles of normal and dystrophic muscle, in order to study fiber size and resting potential, have been done (28). These have shown that there is more variation in fiber size in the dystrophic than in the normal muscle as well as more variation in resting potential. Resting potential is well correlated with fiber size in small fibers. There is more extracellular space in dystrophic muscle. The mean fiber size is twice normal.

The bioelectric properties of dystrophic muscle were studied by Conrad and Glaser (13). They found no significant difference in resting potential of excitable fibers, but a lower average resting potential in dystrophic muscle. Dystrophic muscle had multiple action potentials both spontaneously and in response to stimulation. It also showed increased excitability. The increase in resting potential following reduction of the potassium concentration around the muscle was greater in the dystrophics than in the normals. This is interesting in view of the known decrease in potassium concentration in dystrophic muscle.

Multiple action potentials were more frequent in the low potassium environment.

Conrad and Glaser studied the miniature end plate potentials (mepp) at the myoneural junction in dystrophic muscle. These potentials occur at the motor end plate, and seem to be sub-threshold activity caused by spontaneous depolarization of nerve endings or release of acetylcholine. They found that the rate of miniature end plate potential production was decreased in dystrophic diaphragm and that the response to hypertonic saline was absent. The decay constant of the mepp is longer in dystrophic muscle, but the amplitude of the mepp was not abnormal.

Studies of fatigue in dystrophic muscle indicate a change in transmission of nerve impulses to muscle. Tetanic stimulation leads to a longer tension response and a longer action potential response. Single shock stimulation leads to a tetanic response, a longer decay period, but less tension (14).

Baker et al have shown that neostigmine induces tremors and increases existing tremors in dystrophic mice. A resistance to curare was also noted by them in these mice (3).

Electron microscopic studies have revealed some changes in fine structure in dystrophic mouse muscle (39). Mitochondria are swollen and have a denser matrix. The endoplasmic reticulum is swollen and vacuolization is followed

by destruction. Myofibrils are destroyed. These changes start at random sites. There is no change in the sarcolemmic membrane. The Z-band is intact, which differs from what is seen in an ischemic lesion. There is no apparent change in nuclear fine structure.

Metabolic enzyme concentrations have been determined by homogenization techniques per gram of dry weight and per gram of protein nitrogen (44). Cytochrome oxidase is increased as are the other respiratory enzymes. The glycolytic enzymes remain unchanged. These latter two changes are the opposite of those seen after nerve section. The cathepsins are increased, as they are in any form of muscle wasting.

Studies done on lyophilized muscle in which enzyme concentration was determined by the fluorescence of the oxidized or reduced pyridine nucleotide revealed a increase in the activity of enzymes requiring TPN, add a decrease in enzymes requiring DPN.

The dystrophic strain of mice offers a model of human dystrophy available for study. This is important because it is difficult to do properly controlled studies of human patients with the disease. The mice offer an opportunity for controlled genetic studies, investigation of muscle biochemistry, physiology and microanatomy. Studies in mice may lead to fruitful avenues of investigation in the

human disease. Histological comparisons between mouse dystrophy, Duchenne type dystrophy and human myotomic dystrophy, which the murine disease resemble clinically, have been made by Pearce and Walton (50).

Necrosis of muscle fibers occurs to the same moderate degree in mice as it does in myotonic dystrophy, and it is not as extensive as in the Duchenne types. Fiber enlargement is not as prominent in mice as in Duchenne dystrophy, but again resembles myotonic dystrophy.

Centralization of nuclei occurs in mouse dystrophy as in myotonic dystrophy, and it is absent in Duchenne dystrophy. This trend is opposite to that of prominent nucleoli, which is common to murine dystrophy and Duchenne dystrophy and rare in myotonic dystrophy. Nuclear chains only occur in the murine disease. Sarcoplasmic baslophilia and regenerative phenomena, common to mouse dystrophy and Duchenne dystrophy, are absent in myotonic dystrophy. The increase in fat and connective tissue resembles myotonic dystrophy.

The number of similarities between mouse dystrophy and human disease, particularly myotonic dystrophy point to the usefulness of studying the animal disease with a view toward elucidation of human disease. Obviously, however, the fact that mouse dystrophy is a separate disease taking place in animals must not be forgotten. It cannot

be assumed that any data found in mice apply directly to human disease, but only that the mice can serve as a model on which to base theories of human disease.

C. Effects of Denervation

Changes in both structure and enzyme concentration have been described in muscle following denervation. The first changes noted are in the motor end plates (22). This takes the form of shrinking and breaking up of end plates (9, 10). The changes appear after the degeneration of the nerve endings. Vacuoles begin to appear in the Schwann cells. If innervation is not re-established, atrophy of the muscle fiber with shrinking and fragmentation of the motor end plate and increased connective tissue results.

Enzymes of the phosphatase group have been reported as increased by Golarz and Bourne (19). They found increase in phosphatase activity histochemically.

There has been much disagreement in the literature about the changes in acetylcholinesterase. Coers and Wolf (9) found that cholinesterase persists for a while and then staining becomes paler and irregular. Microchemical techniques reported by Nachmannsohn (22) indicate increases in cholinesterase. This is calculated per gram of muscle, however, and therefore does not take atrophy into account. Others have reported both increases and decreases, depending on the techniques used (22). Snell and McIntyre (40)

found, using the Koelle-Friedenwald technique that cholinesterase gradually disappeared after denervation. There was a continued decrease with time until after 45 days there was none left. Stoerck and Morpeth (reported in 40) extracted whole denervated muscle and did manometric determinations of acetylcholinesterase and found a reduction by one-third, but this did not vary with time.

D. Anticholinesterase Drugs

A number of drugs are known which chemically inhibit acetylcholinesterase. Their action on enzyme activity as demonstrated histochemically is not known.

One such drug is a long-acting anticholinesterase, BC-51. BC-51 is structurally two mestinon molecules linked by a hexamethylene bridge (Figure 3). It was originally introduced by Kraupp (23) and has been used in the treatment of myasthenia gravis (43).

The clinical pharmacology was studied by Kraupp et al (23) in rats. The duration of action was found to be 72 hours. The intravenous ED_{50} for the production of bloody tears was 75.8 micrograms per kilogram; the ED_{50} for weakness was 52.4 micrograms per kilogram. For weakness in all animals, 100 micrograms per kilogram given intravenously was required. The LD_{50}, given intravenously, in rats, was found to be 607 micrograms per kilogram.

II. METHODS AND EXPERIMENTAL DESIGN

A. Design

There are a number of reasons why it seemed worthwhile to study the myoneural junction in this strain of mice. The changes in excitability of muscle and miniature end plate activity suggest that there may be something wrong with the transmission of nerve impulses to the muscle, as does the hypersensitivity to neostigmine and the resistance to curare. The anatomic changes in the muscle are not precisely those of denervation, nor are they precisely those of primary myopathy. It seemed interesting therefore, to study the acetylcholinesterase activity at the myoneural junction in these animals as an anatomic parameter of the nerve to muscle transmission.

It also seemed interesting to study the myoneural junction in denervated mouse muscle, and muscle from mice treated with anticholinesterase medication. These provide known anatomical and physiological lesions to study. A histochemical method was utilized to study the acetylcholinesterase activity at the myoneural junction in all three types of animals: dystophic, denervated, and drug recipient.

The problem of histochemical localization in general is an interesting one. The basis for histochemistry is a

chemical reaction of a substrate at the enzyme site which forms, in a soluble phase, a chemical moiety which will then react with another reagent to form an insoluble product at the enzyme site. This product is preferably colored so that it can be visualized by light microscopy. Techniques for electron microscopy must be modified in order to obtain reaction products that are electron dense.

The precision and accuracy of the technique depends on a number of factors. A physiologic substrate is of course, preferred. Diffusion of the end product leads to imprecise localization. This is best prevented by a technique in which a rapid reaction leads to an insoluble product. Precision is also increased with encreasing size of the enzymatic site. If the reagent reacting with the stain precursor is used in large quantity and the capture reaction is a first order reaction kinetically, better localization will result (20).

It is useful to have a reaction which is suitable for frozen section technique. Better preservation of fine structure is usally a result of frozen section. In addition, many fixatives destroy enzymes, and preservation of enzyme integrity is a requisite to good histochemical technique. Histochemical interpretation is difficult and misleading if the nature of the chemical reactions involved is unknown. Similarly, non-specific reactions lead to mistaken interpretation of histochemical results.

The general priciples of histochemistry can be applied to the specific case of the localization of acetylcholinesterase. Formalin inactivates the enzyme (24), and so it must be used cautiously if at all, even though it may give good histological results. The reactions of this enzyme are pH dependent (24), and pH changes may lead to erroneous results. Cholinesterase is however, well preserved in freezing techniques, and so cryostat sections are useful.

The specificity of esterases is largely dependent on the length of the hydrocarbon chain on either side of the ester linkage. (16). Adams and Whitaker (reported in 16) showed that acetylcholinesterase will hydrolyze choline and non-choline esters. The rate decreases sharply with the addition of chains longer than acetyl. The shape of the molecule is more important than the presence or absence of a quaternary ammonium. Cholinesterase is more specific for choline esters than is acetylcholinesterase. Acetylcholinesterase hydrolyzes analogues nearly twice as fast as does non-specific cholinesterase.

Several techniques for localizing cholinesterases have been described. Early techniques utilized high molecular weight esters of choline and hydrolysis of these was not complete. These techniques domonstrated non-specific cholinesterases (49). The Koelle-Friedenwald technique (31) utilized acetylthiocholine as a substrate. The end product

however, was a coarse, granular, slightly soluble precipitate. This had the disadvantage of relatively high diffusability, and its coarse granularity made microscopic resolution rather poor. Other techniques that were developed (49) had the problem of a substrate that was not specific for the enzyme. Some had end products that were such large crystals that they interferred with cytologic localization.

In 1951, some physicochemical studies by Wilson (47) formed the background work for a histochemical technique later developed by Barrnett and Palade (5). Wilson was studying the mechanism of hydrolysis of esters by acetylcholinesterase. He was using thioacetic acid (CH_3COSH) as a substrate for enzymatic hydrolysis. The reaction consists of acetylation of the enzyme with the simultaneous release of H_2S. The acetylated enzyme then reacts with water to reform the enzyme and produce acetic acid. It is thus, the enzymatic hydrolysis of thioacetic acid. It is analogous to the enzymatic hydrolysis of acetylcholine. The reaction is very much pH dependent. Decreasing pH increases the amount of uncissociated substrate (since it is a weak acid). The rate of the reaction increases with increasing undissociated substrate, since it is undissociated substrate that can react with the enzyme. However, enzyme activity was shown to decrease with decreasing pH. Therefore, the reaction must be

studied at an optimum pH - one low enough that the concentration of undissociated substrate is fairly high, but not no low that the enzyme is destroyed. Wilson also found that prostigmine inhibited this reaction completely.

The reaction was adapted for histochemical use and reported by Barrnett and Palade (5) in 1959. This technique utilizes thioacetic as a substrate for enzyme activity. In the presence of lead nitrate, the liberated H_2S reacts with the lead to form a black lead sulfide precipitate at the site of the enzyme activity. The reaction product, PbS, is a fine, black precipitate which is easily seen and does not disturb cellular detail. It is a heavy, insoluble product which does not easily diffuse. The technique when first used was employed to study enzyme activity at the M-band in striated muscle. These deposits are too small to be seen by the light microscope. The technique does, however allow visualization of the motor end plate by light microscopy and comparison of structure and distribution in normal and experimental animals.

The reaction does not take place in the presence of compounds known to inhibit the activity of the enzyme. Two kinds of enzyme inhibition have been described (33). Competitive inhibition is inhibition of enzyme activity by a compound that reacts in a reversible reaction to form an enzyme-inhibitor complex. There is competition between the

substrate and inhibitor for active enzyme sites. This is dependent on the concentration of inhibitor and of substrate. Inhibition may be overcome by the addition of high concentrations of substrate. The amount of inhibition is dependent on substrate concentration if enzyme concentration is constant.

In non-competitive inhibition, only the concentration of inhibitor is related to the amount of inhibition. The formation of enzyme-substrate complex does not take place at the site of enzyme activity, but at a site which renders the enzyme inactive. The reaction is not reversible.

The two most common types of acetylcholinesterase inhibitors are the structural analogues of acetylcholine, and the organophosphorus derivatives. The structural analogues are adsorbed on both the anionic and esteratic sites of the enzyme. The organophosphorus compounds are only adsorbed at the esteratic site. The structural analogues act completely as competitive inhibitors. The organophosphorus inhibitors seem to act in two steps. The first is thought to be competitive in nature, but the second is irreversible.

In this work, physostigmine, a competitive structural analogue of acetylcholine was used. Physostigmine (Figure 4) is an ancient drug first obtained from the calabar bean which grows in Nigeria. It has been synthesized. Enzyme activity was studied in this work by varying

inhibitor concentrations at known, constant substrate concentrations.

The basic histological and histochemical techniques used were common to most of the animals.

B. Dystrophic Mice

The dystrophic animals studied were dystrophic and littermate controls of the Bar Harbor strain 129 (Dydy), obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor,Maine.

C. Denervated Mice

The denervated muscle studied was obtained from white Swiss laboratory mice. Under ether and nembutal anesthesia, the left sciatic nerve was identified under the gluteus maximus muscle near the point where it leaves the pelvis. Stimulation of this nerve led to movement of the left leg, thus aiding in its identification. The nerve was ligated with black silk, divided, and several millimeters were removed to prevent regrowth. The wound was closed with black silk. Limp and foot drop were immediately noted on the affected side. Atrophy occurred within one or two weeks on the affected side. The mice were studied seven to eight weeks after denervation. The unoperated leg was always studied simultaneously as a control.

D. Drug Recipient Animals

BC-51, a long acting anticholinesterase was kindly supplied by Dr. Paul Hoefer of Columbia University College of Physicians and Surgeons. It was diluted from a solution of 1mg/ml to a solution of 0.2 gm/ml and resterilized. It has been shown to be heat stable.

Two groups of Sprague-Dawley rats were studied. One group was treated every 72 hours with BC-51, 0.1mg/kg given intramuscularly. The other group was treated with an equivalent volume of normal saline in the same way. The animals were studied at several week intervals. One animal was given a lethal overdose (1.2 mg/kg) to demonstrate the efficacy of the drug. The animal succumbed in a typical cholinergic crisis five hours after the drug was given, with convulsions, profuse diaphoresis, salivation, respiratory distress, bloody tears, and cardiac arrest.

Normal Swiss laboratory mice and denervated mice were also given BC-51, 0.1mg/kg, intramuscularly every 72 hours. They were studies after seven and eight weeks of treatment.

The animals were sacrificed by mild etherization followed by cervical fracture until ether was shown not to affect the results even when used in lethal quantities. Thereafter ether alone was used.

E. Histological Techniques

Tibialis anticus muscle was studied in all cases, and was dissected from the leg immediately after sacrifice. In the early studies the dissected muscles were tied to plastic rectangles with surgical silk in order to prevent shrinkage and fixed for eight to ten hours, in cold 10% Formalin in 0.1 M Sorenson's phosphate buffer (11, 36) at pH 7.4 containing 0.4 M sucrose. The sucrose was used to raise the osmolar concentration of the medium and thereby preserve the fine structure of the muscle fibers (5). The histochemical reaction was carried out, and then the muscle was sectioned at 20-50u on an A/O Spencer freezing microtome, and stained in the usual way with hematoxylin and eosin. Later studies were carried out without fixation prior to the histochemical reaction. These muscles were rapidly frozen in small pieces in liquid nitrogen and sectioned at 8u with the Slee cryostat. After the histochemical reaction was done, the sections were fixed while on coverslips in 10% Formalin for severals hours, then stained with hematoxyline and eosin and mounted on glass slides. End plates were measured with a Carl Zeiss eyepiece micrometer calibrated with a Bausch and Lomb stage calibrator.

F. Histochemical Techniques

The histotchemical localization was carried out by the method described by Barrnett (5) in the following way.

Thioacetic acid (Eastman) 0.25ml, was titrated to pH 6.4 with 1.0 and 0.1N NaOH (Mallinckrodt). The pH determinations were originally done with a Beckman Model 180 pH meter, and later with a Corning pH meter, which proved to be more accurate. The volume was then adjusted to 20ml. with sodium cacodylate buffer made to pH 6.4 (38). Then 48 mg. of $Pb(NO_3)_2$ (Mallinckrodt) in 5 ml. of cacodylate buffer were added slowly with stirring to complete the incubation solution. The pH was rigidly measured, because above pH 6.8 slow spontaneous hydrolysis of the substrate occurs, and at pH 7.4 it occurs at an appreciable rate. Low pH of course, destroys the enzyme. When whole muscle was used, the reaction was observed with a dissecting microscope. Control and experimental muscle were always done together under identical conditions. Muscles were studied using the same incubation time, and also varying the time until the experimental end plates reached the same darkness as the controls, as observed under the dissecting microscope.

It has been shown by Barrnett (4, 5) that the reaction does not occur without the substrate.

G. Inhibition Studies

Inhibition studies were done using solutions of physostigmine salicylate (Mallinckrodt) in varying concentrations. The whole muscle was incubated in inhibitor for fifteen minutes, the sections for twenty minutes. Control and experimental muscle, inhibited and non-inhi-

bited, were all run simultaneously. During incubation of the inhibited tissues, the reaction solution contained the same concentration of inhibitor as did the inhibiting solution. This maneuver was used because physostigmine is known to be a competitive inhibitor.

III. RESULTS

A. Dystrophic Mice

Dystrophic muscle shows the classical characteristics as described by Michelson. The muscle fiber size varies greatly, there is coagulation necrosis, nuclear rowing, and increased connective tissue. The end plates vary in shape and appear "motheaten".

In the whole muscle studies, after incubation for the same length of time, end plates appeared to be much lighter. It took 1.5 times as long for them to achieve the same darkness as the controls. The normal pattern of distribution was not obvious. However, derangement of the fibers with coagulation necrosis occurs, and the absence of the pattern may well be due to this. In the normal muscle, the size of the end plates follows a normal distribution curve. In the dystrophic muscle, however, there seems to be two populations of end plates: one of end plates the same size as the normals, and one of end plates more than twice the size of normals (figure 5).

When a concentration of $1x10^{-5}M$ Physostigmine was used for inhibition, no end plates were seen in either the normal or the dystrophic muscle. In all cases of normal and dystrophic muscle run simultaneously without inhibitor, end plates were seen.

When the reaction was done in the presence of a physostigmine concentration of $1x10^{-7}M$, the number of normal control end plates that could be seen was reduced to about one third its original value, while the number of dystrophic end plates was reduced to less than 1/12 its original value (Table 1). Those remaining were light and difficult to see.

In the cryostat sections, the same subjective darkness was achieved in normals and dystrophics after twenty minute incubations.

Total inhibition of normal control muscle required $1x10^{-6}M$ physostigmine solutions. Histochemical reactions occurred at concentrations lower than that, and end plates could easily be seen by light microscopy. To inhibit completely the histochemical reaction in dystrophic muscle, however, only a concentration of $1x10^{-8}M$ physostigmine was required, a difference one hundred fold. Histochemical reaction occurred in dystrophic muscle at $1x10^{-9}M$ physostigmine. It was not observed at $1x10^{-7}M$. (Table 2) (See photomicrographs)

B. Denervated Mice

The animals that had been denervated showed marked muscle atrophy and weakness. The muscle had shrunk to one half its normal size. The sciatic nerve was still severed and ligated. Microscopically, the muscle fibers were small, varied in size and shape, and separated by a marked increase in connective tissue. End plates varied in size and shape, and were less regular in outline and less darkly stained (See photomicrographs). It only required inhibition concentrations of $1x10^{-9}M$ to inhibit the histochemical reaction at these end plates. Normals are not inhibited by concentrations less than $1x10^{-6}M$.

C. Drug Recipient Animals

After two weeks the histochemical reaction in the drug recipient rats was inhibited by concentrations of $1x10^{-7}M$ physostigmine, whereas in the control rats it was not. After eight weeks, however, neither the reaction in the drug recipient rats nor the reaction in the controls was inhibited by concentrations of $1x10^{-7}M$ physostigmine.

The denervated mice after eight weeks did not return to normal cholinesterase activity. The histochemical reaction was inhibited by $1x10^{-8}M$ physostigmine in the denervated non-drug recipient mice. It was not inhibited in normal controls until a concentration of $1x10^{-6}M$ physostigmine was reached. This difference of 1000 fold could

not be accounted for by a weight difference of one half. In the denervated drug recipient animals, no cholinesterase could be stained, even in the absene of inhibitor. In con-denervated drug recipient animals the reaction was inhibited by $1x10^{-7}M$ physostigmine concentrations, but not by $1x10^{-8}M$.

IV. CONCLUSIONS

A. Dystrophic Animals

The histopathological changes in the dystrophic animals are exactly similar to the ones noted by Michelson, Russell, and Harman, and described earlier. However, the histochemical ones shown have not been described. There are two populations of end plates in the dystrophic muscle: one of normal size and one whose average size is 2.1 times normal. The larger end plates are fewer in number. This correlates well with the variation in muscle fiber size, and is probably a reflection of early disintegration of fibers and end plates. However, since immature end plates are often larger than normal, this size variation could be a reflection of lack of normal development in these end plates.

The fact that more time in the reaction solution is required to achieve the same degree of reaction in dystrophics as in normals is explainable in two different ways:

either there is less acetylcholinesterase present in the dystrophic end plate or there is a decreased turnover number of the enzyme in the dystrophic animal. It is hard to explain why the enzyme, which is similar to normal enzyme in many other ways should have a decreased turnover number or rate of reaction. Evidence for the hypothesis that there is less enzyme at the dystrophic end plate is found in the inhibition studies. Since the inhibitor is a competitive one, if substrate and inhibitor concentration are held constant, the amount of reaction is a reflection of enzyme activity. The difference of 100 times in amount of physostigmine required to inhibit the reaction in dystrophic muscle is best explained by a reduction of enzyme activity to 1/100 normal in the dystrophic animals. This difference cannot be accounted for on a weight basis. A reduction in acetylcholinesterase activity at the motor end plate in dystrophic animals correlates well with results reported earlier. If not enough enzyme is present to destroy the acetylcholine produced with motor nerve stimulation, tetanic contractions will be produced, as they have been. The increased excitability and multiple action potentials reported could also be accounted for by a decrease in enzyme concentration. This is also true for the increased decay constant of the miniature end plate potential. Certainly the increased sensitivity to neostigmine

and the resistance to curare reported by Baker are explainable by a decrease in acetylcholinesterase concentration. These studies indicate that a disease process is occurring at the motor end plate in dystrophic mice. There is both anatomic and biochemical degrangement.

B. Drug Recipient and Denervated Mice

The studies in the denervated and drug recipient mice are of a preliminary nature. However, the experiments using drug recipient rats indicate that for some weeks after chronic anticholinesterase administration, the amount of acetylcholinesterase as determined by histochemical methods is less than normal. However, after eight weeks of administration, the amount seems to have returned to normal levels. This is interesting in light of the fact that in patients with myasthenia gravis who are receiving anticholinesterase medications, dosages often need to increased after short term chronic administration to obtain the same clinical effect. The remaining question is that of how inhibition of enzyme activity leads to the production of increased enzyme activity.

The denervated animals show derangement of end plate structure as well as decrease in enzyme concentration as evidenced by the fact that inhibition requires 1/1000 the concentration of inhibitor that normals do. This was

true up to eight weeks after denervation, the length of time these animals were studied. The denervated animals who received anticholinesterase medication, unlike the non-denervated animals, were unable to return to the original levels of enzyme activity. It appears that an intact nerve is necessary for the production of acetylcholinesterase. The denervated animals are more vulnerable to the actions of anticholinesterase drugs.

V. SUMMARY

The acetylcholinesterase activity at the motor end plate of dystrophic muscle, denervated muscle, and anticholinesterase-treated muscle was studied histochemically using the thioacetic acid technique. Inhibition studies using varying concentrations physostigmine salicylate, a competitive inhibitor.

1. The dystrophic muscle was found to have two populations of end plates: one of normal size and one of over twice normal size.
2. More time was required to complete the reaction in dystrophic muscle and less inhibitor required to inhibit the reaction in dystrophic muscle, indicating a decrease in enzyme activity. The difference in inhibitor concentration was 100 fold.

3. The drug treated animals were found to have decreased enzyme activity for a short time after treatment was begun; they returned to normal activity after eight weeks, indicating some form of regeneration of enzyme activity.
4. Denervated animals were found to have 1/1000 the enzyme activity eight weeks after nerve section.
5. In denervated animals receiving anticholinesterase drugs for eight weeks, no enzyme activity could be seen.

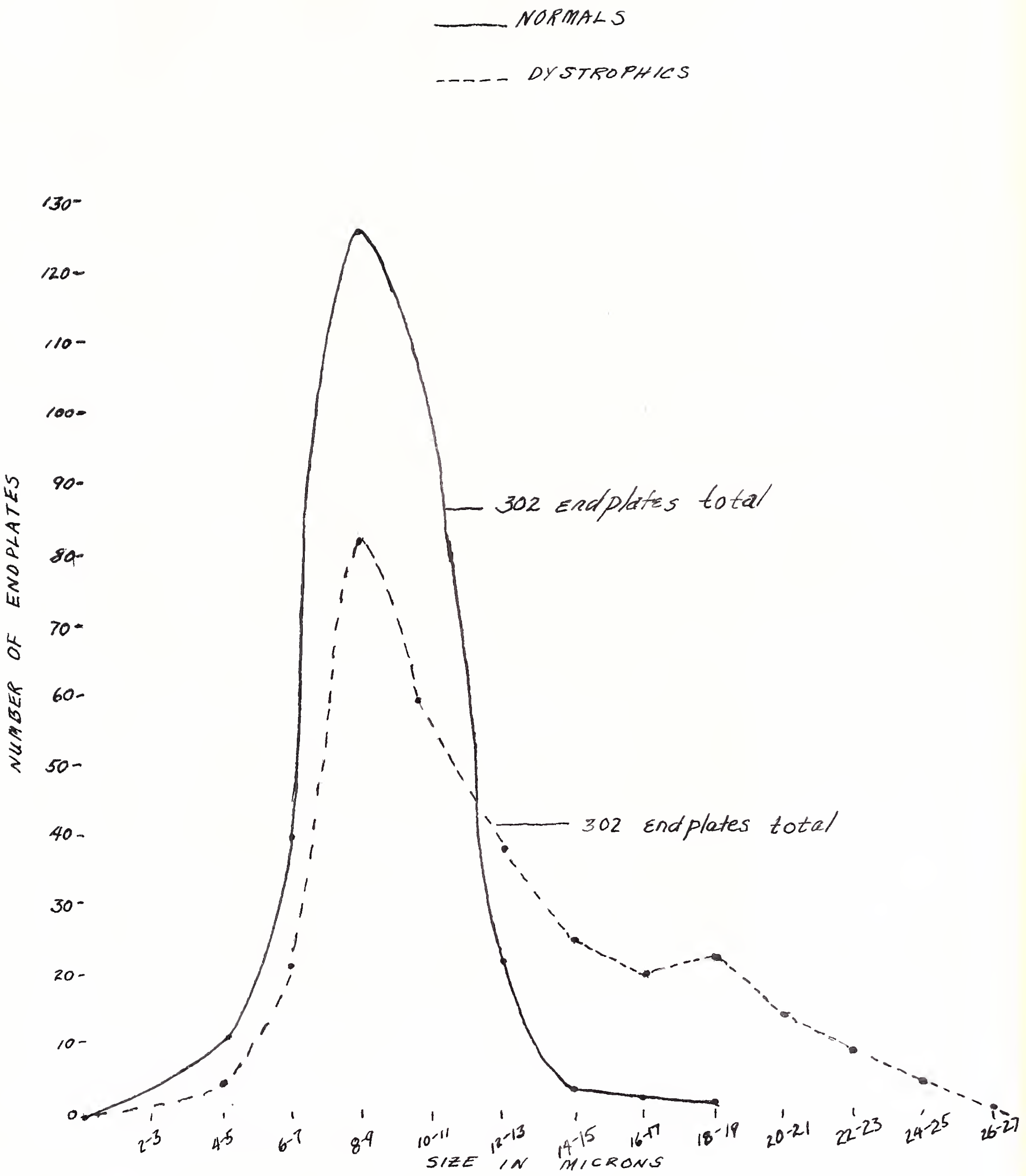

NORMALS
DYSTROPHICS
NUMBER OF ENDPLATES
130
120
110
100
90
80
70
60
50
40
30
20
10
0
302 endplates total
302 endplates total
2-3
4-5
6-7
8-9
10-11
12-13
14-15
16-17
18-19
20-21
22-23
24-25
26-27
SIZE IN MICRONS

FIG.5

Table 1

Physosstigmine Salicylate $1x10^{-7}$Molar

Muscle	No. Sections	No. Endplates	Endplates/Section
Dystrophic Inhibited	14	48	3.4
Dystrophic Uninhibited	13	552	42.4
Control Inhibited	13	287	22.0
Control Uninhibited	10	650	65.0

Table 2

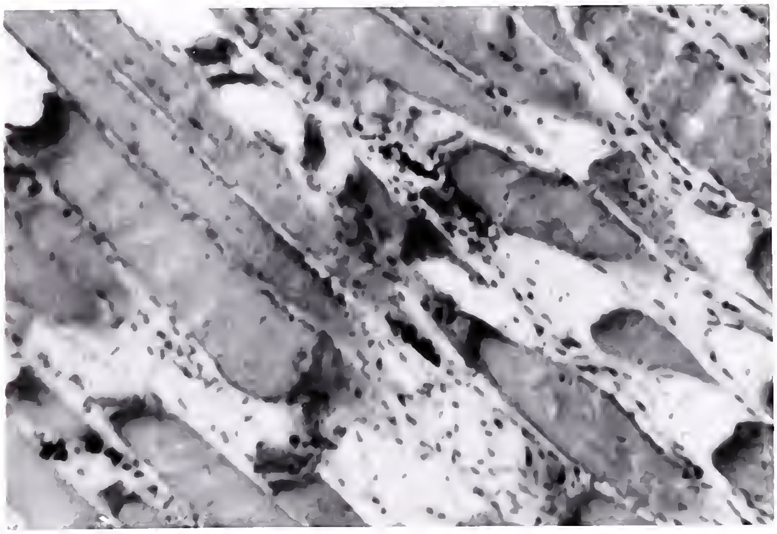
Physostigmine Salicylate

Concentration	Dystrophic Muscle	Normal Muscle
$1x10^{-6}M$	Inhibited	Inhibited
$1x10^{-7}M$	Inhibited	Not Inhibited
$1x10^{-8}M$	Inhibited	Not Inhibited
$1x10^{-9}M$	Not Inhibited	Not Inhibited

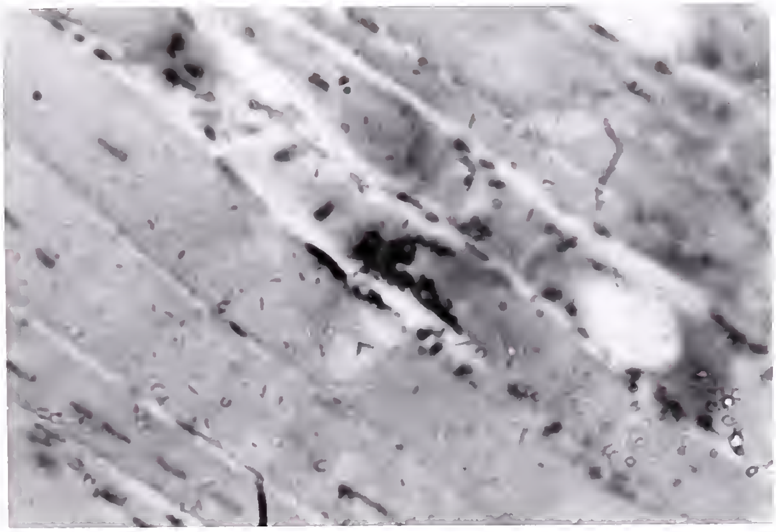
Table 3

Physostigmine

Concentration	Control	Denervated, Drug	Non Denervated, Drug	Denervated, No Drug
$1x10^{-6}M$	Inhibited	Inhibited	Inhibited	Inhibited
$1x10^{-7}M$	Not Inhibited	Inhibited	Inhibited	Inhibited
$1x10^{-8}M$	Not Inhibited	Inhibited	Not Inhibited	Inhibited
$1x10^{-9}M$	Not Inhibited	Inhibited	Not Inhibited	Not Inhibited
No Inhibitor	Not Inhibited	Inhibited	Not Inhibited	Not Inhibited

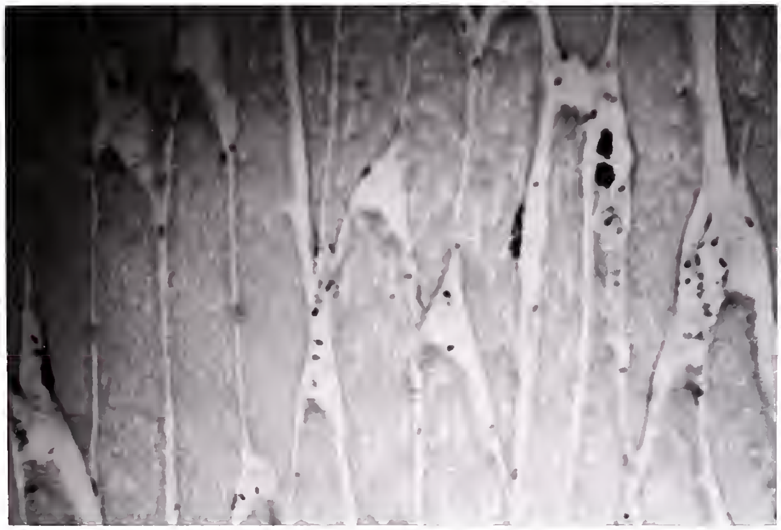


Photomicrograph 1. Dystrophic muscle, no inhibitor. Note coagulation necrosis, nuclear rowing, increased connective tissue, round cell infiltrate, and "moth eaten" endplates. 125x.

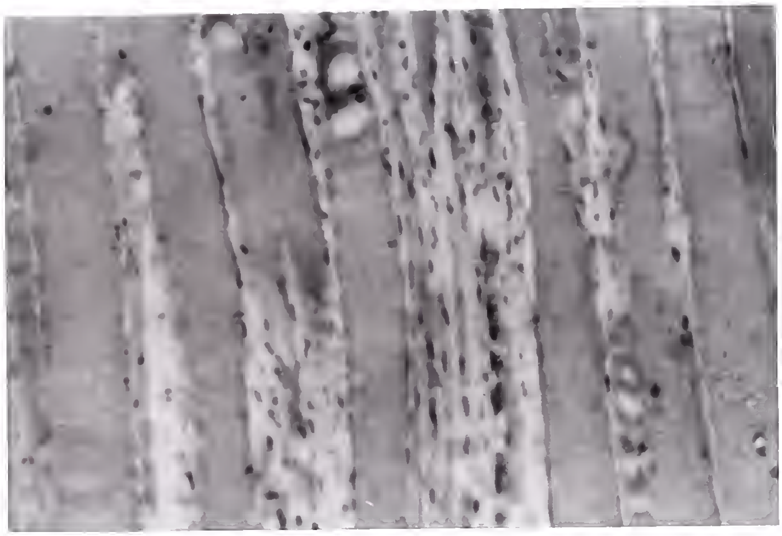


Photomicrograph 2. Dystrophic muscle, no inhibitor. 240x.

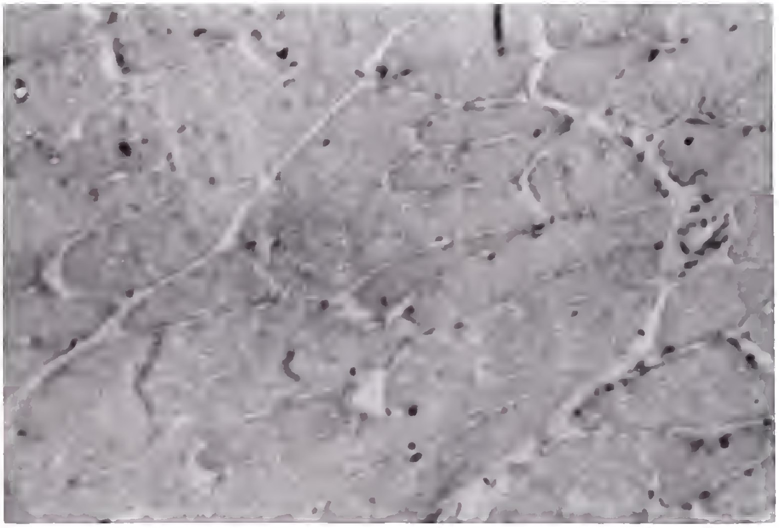
Photomicrograph 3. Dystrophic muscle, 1×10^{-8}M physostigmine. No reaction is seen. 125x.



Photomicrograph 4. Dystrophic control muscle, 1×10^{-8}M. physostigmine. Reaction has occurred. Typical dystrophic lesions are absent from control. 125x.

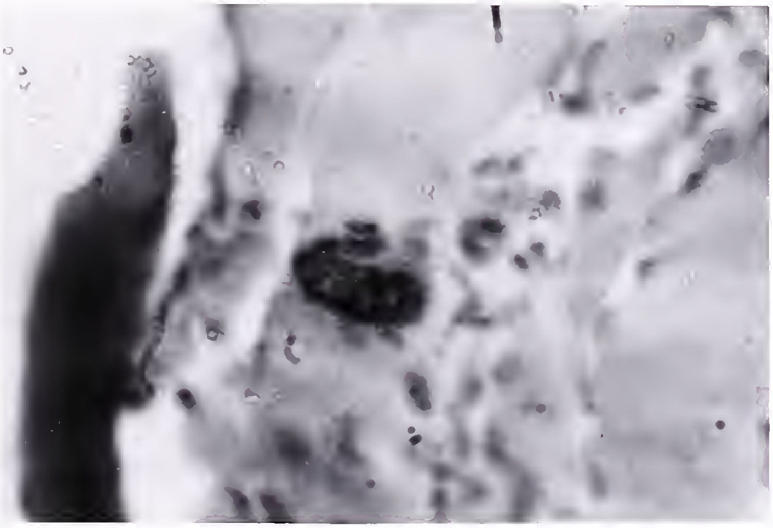


Photomicrograph 5. Dystrophic muscle, 1×10^{-7}M. physostigmine. No reaction has occurred. 125x



Photomicrograph 6. Dystrophic control muscle, 1×10^{-6}M physostigmine. No reaction has occurred. 125x

Photomicrograph 7. Normal mouse muscle, $1x10^{-9}M$ physostigmine. 125x



Photomicrograph 8. Denervated mouse muscle, no inhibitor. Note atrophy, variation in fiber size, and partially destroyed endplate. 440x.

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